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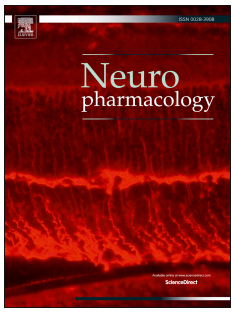

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Perinatal fluoxetine exposure changes social and stress-coping behavior in adult rats housed in a seminatural environment

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Short title: Behavioral effects of perinatal fluoxetine exposure

Keywords: antidepressant, fluoxetine, perinatal, social behavior, sexual behavior, rats, seminatural environment

Abstract

The use of selective serotonin reuptake inhibitors (SSRI) during pregnancy has increased tremendously, but the consequences for the offspring remain largely unclear. Several studies have described potential effects of perinatal SSRI-exposure on neurobehavioral outcomes using simplified rodent test set-ups, however these set-ups only assess a small fraction of the behavior. For translational purposes it is important to take the environmental influences into account which children are exposed to in real life. By using a seminatural environmental set-up, this study is the first to assess behavioral outcomes in offspring exposed to perinatal SSRI exposure under seminatural circumstances. Mothers received daily the SSRI fluoxetine (FLX, 10 mg/kg p.o.) or vehicle (CTR) from gestational day 1 until postnatal day 21. To assess the effect of FLX exposure during early development, female and male offspring were behaviorally tested in the seminatural environment at adulthood. Baseline behavior was measured in addition to responses during and after stressful white-noise events. Behavior was observed on two days, day 4 on which females were sexually non-receptive, and day 7, on which females were sexual receptive. Perinatal FLX exposure reduced general activity in females and increased behavior related to a social context in both males and females. After a stressful white-noise event some behaviors switched. Whereas FLX-females switch from resting socially to resting more solitarily, FLX-males show an increase in self-grooming behavior after the stressor and showed more freezing behavior in the open area. We conclude that perinatal FLX exposure leads to alterations in social and stress-coping behaviors in adulthood, when observed in a seminatural environment. Whether these adaptations in behavior are advantageous or disadvantageous remains to be established.

1. Introduction

Depressive symptoms frequently occur during pregnancy and can affect the developing child in a profound way. Over the last years, selective serotonin reuptake inhibitors (SSRIs) have gained acceptance as medication during pregnancy, which resulted in an increase in the prescription rate in pregnant women (Alwan et al., 2011; Ververs et al., 2006). However, antidepressants can cross the placenta and are present in breast milk (Kristensen et al., 1999; Rampono et al., 2004). As a result, a growing number of children is being exposed to SSRIs during the perinatal period (Kim et al., 2006; Noorlander et al., 2008).

By blocking the serotonin transporter (SERT), SSRIs inhibit the reuptake of serotonin (5-HT) into the presynaptic nerve terminals, which results in an increase in the synaptic concentration of 5-HT. During adulthood, 5-HT mainly acts as a modulatory neurotransmitter regulating emotion, stress responses, sleep, learning, cognition, and attention (Canli and Lesch, 2007). During early brain development, on the other hand, 5-HT also acts as a neurotrophic factor, regulating cell division, differentiation, migration, and synaptogenesis (Azmitia, 2001; Gaspar et al., 2003). Therefore, it is assumed that changes in 5-HT levels during *in utero* neurodevelopment have the potential to affect these processes as well as subsequent serotonergic function and vulnerability to affective disorders (Lesch and Mossner, 1998).

Several studies in humans have described an effect of antenatal SSRI-exposure on neurobehavioral outcomes. For example, SSRI treatment during pregnancy has been associated with disturbed sleep patterns, affected social-emotional development, and increased internalizing and externalizing behavior in the offspring (Brandlistuen et al., 2015; Oberlander et al., 2010; Weikum et al., 2013). Recently, an increased risk for autism spectrum disorder (ASD) in offspring was added to this list (Boukhris et al., 2016; Rai et al., 2013). ASD can be characterized by e.g. difficulties in social interaction and communication and a tendency to engage in repetitive

behaviors. The problem with these human studies, though, is the difficulty to discern between the effects of the SSRIs and the effects of the mothers' underlying depression. In fact, when controlled for maternal mood and stress, this link between antenatal SSRI use and the occurrence of ASD in the offspring does not prevail (Brown et al., 2017). Still, it is difficult in human studies to control for all potential environmental influences. Animal models, on the other hand, can be used to study the effects of SSRI use on the neurodevelopmental outcomes in the offspring without interference of potential confounders.

Several studies have shown that SSRI exposure during development can alter social behavior: in juvenile rats, social play behavior with an unfamiliar play partner is reduced after perinatal SSRI exposure, (Houwing et al., 2019b; Khatri et al., 2014; Olivier et al., 2011b; Rodriguez-Porcel et al., 2011; Simpson et al., 2011). Furthermore, SSRI exposure throughout pregnancy and lactation can increase aggressive behavior in adult male mice (Kiryanova and Dyck, 2014; Svirsky et al., 2016), while postnatal SSRI exposure has the potential to reduce sexual behaviors in rodents (Gouvea et al., 2008; Harris et al., 2012; Rayen et al., 2013; Rodriguez-Porcel et al., 2011). Unfortunately, there are still a lot of discrepancies between the different studies, some of which can be explained by the timing of the SSRI exposure. In the adolescent and adult brain the SERT is only expressed in neurons of the raphe nucleus, but at early developmental stages the SERT expression pattern is more widespread (Homberg et al., 2010; Olivier et al., 2011a). Altered activation of these transporters, and thus the serotonergic tone, could lead to changes in brain development. Besides, although the transient SERT expression disappears during the early postnatal phase, 5-HT retains its neurotrophic actions. It has been suggested that inhibition of SERT and excess 5-HT exposure during a critical period in fetal development leads to alterations in monoamine systems throughout various brain regions and results in long lasting neurological effects which differ throughout lifespan (Homberg et al.,

2010; Weinstock, 2015). While some studies exposed the offspring to SSRIs prenatally, others used a postnatal approach. The different timing of the SSRI exposure could, in theory, induce different behavioral outcomes due to the different patterns of SERT expression (Ansorge et al., 2004; Popa et al., 2008).

In our current experiment, we circumvented the different potential outcomes of SSRI exposure on critical time points, by administering pregnant females daily with fluoxetine (SSRI) or vehicle from gestational day 1 (GD1) until the pups are weaned at postnatal day (PND) 21. This timeframe was chosen to resemble the entire human pregnancy period and part of the postnatal period, since rat brain neurodevelopment at postnatal days 1–10 equals the third trimester of pregnancy in humans (Andrews and Fitzgerald, 1997; Dobbing and Sands, 1979). Thus, we were able to investigate the neurodevelopmental effect of SSRI treatment during pregnancy on the offspring in a way that is translational to the human situation.

To bypass another limitation of previous studies, our experiment used a seminatural environmental set-up in which rats live in groups for several days and can express all aspects of their natural behavior (Bove et al., 2018; Buwalda et al., 2017; Le Moëne and Ågmo, 2018, 2019). This way, the behavioral alterations in the offspring due to perinatal SSRI exposure can be investigated in a social context, in which the consequences of environmental influences and life-events can be determined. Simplified rodent test set-ups can only investigate a small fraction of the behavior and fail to take into account the environmental influences children are exposed to in real life. The social interaction test, for example, investigates the time two paired rats sniff and groom each other, as an indicator of social behavior. However, the rats are paired in a small controlled test arena which does not allow them to escape from the situation. In real life, people can decide to (socially) interact with one or another, or simply withdraw from social interaction. Environmental factors can influence the decisions that are being made at that moment. Therefore,

the seminatural environmental approach used in our study is a more translational test set-up in which the full repertoire of behavior can be expressed and investigated.

Our test approach allows to study the same group of rats (cohort) over time in a seminatural setting without a change in test environment (e.g. the transport from homecage to test cage is stressful by itself). In order to investigate the consequences of experiencing stressful life-events, we simulated such an event in the seminatural environment by exposing the rats to a 10-minute lasting 90 dB white-noise episode. White-noise is comparable to the sounds of the natural predator of rats, rattlesnakes, which induces physiological and behavioral responses associated with stress (Rowe et al., 1986; Weyers et al., 1994). By doing so, we were able to investigate the behavioral adaptation caused by perinatal SSRI exposure on baseline levels in combination with studying the behavioral changes during and after a stressful event. In addition, because the hormonal status of females can have an effect on their behavior, we controlled for the estrous cycle of females. We observed the behavior of both males and females before, during and after the stressor on a day with females in diestrus and on a day when in proestrus (induced with hormonal treatment). We hypothesized that perinatal SSRI exposure would reduce components of social behavior in the offspring based on results found in simplified rodent tests (Khatri et al., 2014; Olivier et al., 2011b; Rodriguez-Porcel et al., 2011; Simpson et al., 2011; Zimmerberg and Germeyan, 2015). During and after the stressor, we expected that FLX exposed animals would display increased freezing behavior based on the increased anxiety-levels found in rats in classic tests assessing anxiety-like behaviors (Olivier et al., 2011b). As sex differences are prominent after early-life events, we assess both males and females and expect to find differences in the responses to the perinatal SSRI treatment, where responses in males are more robust than in females based on results found in simplified rodents tests (Houwing et al., 2019b).

2. Material and Methods

2.1 Animals and dam housing conditions

A total of ten female and ten male Wistar rats (weighing 200-250 g at the time of arrival) were obtained from Charles River (Sulzfeld, Germany) for breeding. They were used as dams and potential father of the offspring. These animals (but also the future offspring) were housed in same sex pairs in Makrolon® IV cages in a room with controlled temperature (21 ± 1 °C) and humidity (55 ± 10 %) on a 12:12 h light/dark cycle (lights on 11:00 h). Commercial rat pellets (Standard chow from SDS, Special Diet Services) and tap water were provided ad libitum, and nesting material was presented.

All experimentation was carried out in agreement with the European Union council directive 2010/63/EU. The protocol was approved by the National Animal Research Authority in Norway.

2.2 Breeding and antidepressant treatment

Prior to breeding, females were checked daily for their estrus cycle stage by placing them together with a male rat for maximum 5 minutes. They were considered receptive when they responded to a mount with a lordosis response. When receptive, the females were placed with a male for approximately 24 hours (Gestational day 0). During this period, each female-male couple was housed in a Makrolon® IV cage. After 24 hours, both male and female returned to their original homecage (with same-sex partner) for the first two weeks of pregnancy. On gestational day 14, the females were housed singly in Makrolon® IV cages with access to nesting material.

From gestational day 1 (G1) until postnatal day 21 (PND21), females were administered daily with either 10 mg/kg fluoxetine (apotekproduksjon, Oslo, Norway) or a vehicle

(Methylcellulose 1%, (Sigma, St. Louis, MO, USA)) using gavage with a stainless steel feeding needle (total of 6 weeks). Fluoxetine tablets (for human usage) were pulverized and dissolved in sterile water (2mg/mL) and injected at a volume of 5mL/kg. As control condition, methylcellulose, the non-active filling of a fluoxetine tablets, was dissolved in sterile water to create a 1% solution and administered at a volume of 5mL/kg as well. The amount of vehicle/fluoxetine given was adjusted upon the weight of the females who were weighed every three days. The dose of fluoxetine was based on comparison to human situations (Lundmark et al., 2001; Olivier et al., 2011b). Near the end of pregnancy, dams were checked twice a day (9:00 h and 15:00 h) for pup delivery.

2.3 Offspring housing conditions before the seminatural environment

After birth, litters were not culled. Pups were weaned at PND 21 and housed in groups of two or three same sex littermates in Makrolon IV cages (see Table S2 for more details). Ears were punched for individual recognition. Until introduction into the seminatural environment (at 13-18 weeks of age), offspring were left alone and only handled during weekly cage cleaning. Only female rats were “disturbed” for the ovariectomy surgery two weeks before introduction to the environment (see 2.4).

2.4 Ovariectomy surgery

Female offspring were ovariectomized to be able to control their estrous cycle with hormone injections. This allowed us to 1) control for the hormonal state (diestrus versus proestrus) when exploring the effects of perinatal SSRI exposure in females, and 2) to induce sexual receptivity on day 7 to study the effects on sexual behavior, and 3) to limit interference of copulation (a behavior often dominant to other behaviors) on the other days.

Females were given isoflurane anesthesia and were placed on their ventral surface. In addition, buprenorphine (.05 mg/kg) and Carprofen (5mg/kg) were given subcutaneously in the upper neck region of the animal before surgery. Ovariectomy was preceded by a 1-2 cm longitudinal midline dorsal skin incision at the lower back of the animal. Muscle incisions were made bilaterally and the peritoneal cavity was accessed. The ovary was located, the connection between the fallopian tube and the uterine horn ligated, and the ovary was extirpated. Muscle incisions were sutured and a wound clip was placed for skin closure. Animals were given Carprofen (5mg/kg subcutaneously) 24 and 48 hours after surgery. Female offspring were singly housed for 3 days during recovery before returning to their homecage.

2.5 Design

For the behavioral observations, five cohorts of eight rats (offspring) were used, with one cohort in the seminatural environment at the time, thus using 5 different cohorts. A cohort of rats consisted of four males and four females, each sex consisting of two rats from control mothers and two from fluoxetine treated mothers. This resulted in ten animals per treatment group coming from 5 batches for data analysis; 10 females and 10 males that were exposed to fluoxetine during development (FLX-females and FLX-males, respectively), and 10 females and 10 males that were exposed to vehicle during development (CTR-females and CTR-males, respectively).

Within a cohort, same sex animals came from different litters. However, within a cohort, almost every animal had 0-1 sibling from the opposite sex ((see TableS1 for more details)), due to a limited amount of litters available. These littermates, however, were housed in different home cages after weaning. Animals were otherwise unfamiliar to each other and sexually naive.

2.6 Procedure

The day before introduction to the seminatural environment (see 2.7 for description of the environment), offspring were shaved and marked under isoflurane anesthesia for individual recognition on video (the wound clips of the females were also removed at the same time). For both sexes, a square area of approximately 4 x 4 cm was shaved either on the upper back/neck, middle back, lower back or the animal was not shaven at all. In addition, the tails of the females were marked with either 1, 2, or 3 rings (0.5 cm) around the base of the tail using a permanent black marker. Female number four received staining at the tip of the tail (approximately 3 cm). The males received the same markings but the rings were broader (about 1 cm) and male four received an extra ring below the marking of the tail tip. In addition, body weight of the animals was measured. No differences in bodyweight were found between CTR-rats and FLX-rats at this moment.

The offspring were placed in the seminatural environment for 8 days (day 0 – day 8) when adult. Since offspring were entering the seminatural environment in cohorts, the age varied between 13 to 18 weeks. An overview of the whole procedure from the beginning of antidepressant treatment until the end of testing of the offspring is given in Figure 2. Each cohort of animals was introduced on the first day (Day 0) at 10:00 h by placing first the females followed by the males in the open field. On day 8, the animals were taken out from the burrow system at 10:00 h, the end of the experiment. After removal, animals were weighed again (again no significant differences between CTR-rats and FLX rats), and underwent whole animal perfusion fixation. Brains were removed and stored for potential further analysis (not included in this study).

Hormone injections were administered to the females on day 5 (estradiol benzoate) and day 7 (progesterone) at 10:00 h (See 2.8 for more details). During the experiment the seminatural

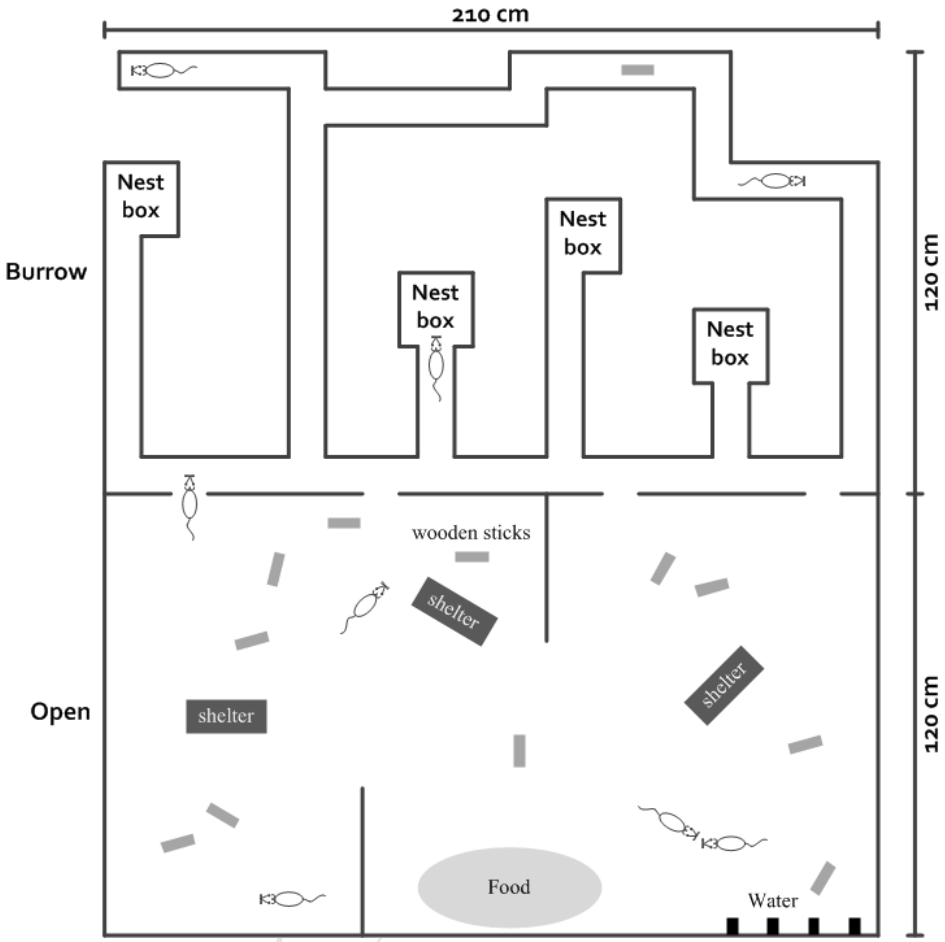
environment was not cleaned, but between colonies, the seminatural environment was thoroughly cleaned to remove olfactory cues from previous animals.

2.7 Seminatural environment

The seminatural environment (2.4 x 2.1 x 0.75 meters) consisted of a burrow system and an open field area which were connected by four 8 x 8 cm openings (Figure 1) (Chu and Agmo, 2014; Snoeren et al., 2015). Several tunnels (7.6 cm wide and 8 cm high) and four nest boxes (20 x 20 x 20 cm) were present in the burrow system. The burrow system was covered with Plexiglas while the 75cm high open area was left open. The open area also had two partitions (40 x 75 cm) to create obstacles simulating the nature. Even though the animals were able to move freely between the open area and burrow system, a curtain between the arenas allowed the light intensity for both arenas to be controlled separately. While the burrow system remained in total darkness for the complete day, a day-night cycle was simulated in the open area. A lamp 2.5 m above the center of the open area provided light (180 lux) from 22:45 h to 10:30 h (simulating day light). From 10:30 h to 11:00 h the light intensity gradually decreased to approximately 1 lux, the equivalent of full moonlight. Similarly, the light gradually increased again from 1 to 180 lux from 22:15 h to 22:45 h.

Both the open area and the burrow system were covered with a 2 cm layer of aspen wood chip bedding (Tapvei, Harjumaa, Estonia) and nest boxes were provided with 6 squares of nesting material each (nonwoven hemp fibers, 5 x 5 cm, 0.5 cm thick, Datesend, Manchester, UK). In the open area 3 red polycarbonate shelters (15 x 16.5 x 8.5 cm, Datesend, Manchester, UK) were placed and 12 aspen wooden sticks (2 x 2 x 10 cm, Tapvei, Harjumaa, Estonia) were randomly distributed. Food was provided in one big pile of approximately 2 kg, in front of the

264 open area wall opposite of the openings. Water was available *ad libitum* in four water bottles
265 located in the lower right corner of the open field.
266
267 **Figure 1. Overview of the seminatural environment**



268
269
270 Video cameras were mounted on the ceiling 2 m above the seminatural environment: one
271 above the open field (Basler) and an infrared video camera above the burrow system (Basler).
272 Videos were recorded using Media Recorder 2.5. Cameras were connected to a computer and
273 data was (immediately) stored on an external hard drive. Every 24 h, the recording was manually
274 stopped and restarted to create recordings with a length of 24h. This was done to make sure that

when a recording error should occur during the 8 day period, only one recording day would be lost.

2.8 Hormone treatment

During the experiment, female rats were shortly taken out of the seminatural environment on day 5 and 7 in order to receive a subcutaneous hormone injection. The ovariectomized females received 18 µg/kg estradiol benzoate on day 5, and 1 mg of progesterone on day 7. Injections were given at 10:00 h and females were placed back at the same place into the burrow part of the seminatural environment. Since the males did not receive any hormone injections, they were left undisturbed in the seminatural environment. The doses of estradiol and progesterone were based on previous research showing that it produces maximal receptivity and high intensity of female reproductive behavior (see (Spiteri et al., 2010)).

Estradiol benzoate and progesterone (Sigma, St Louis, MO, USA) were dissolved in peanut oil (Apoteksproduksjon, Oslo, Norway) and injected in a volume of 1 ml/kg.

Figure 2. Schematic overview of all experimental procedures

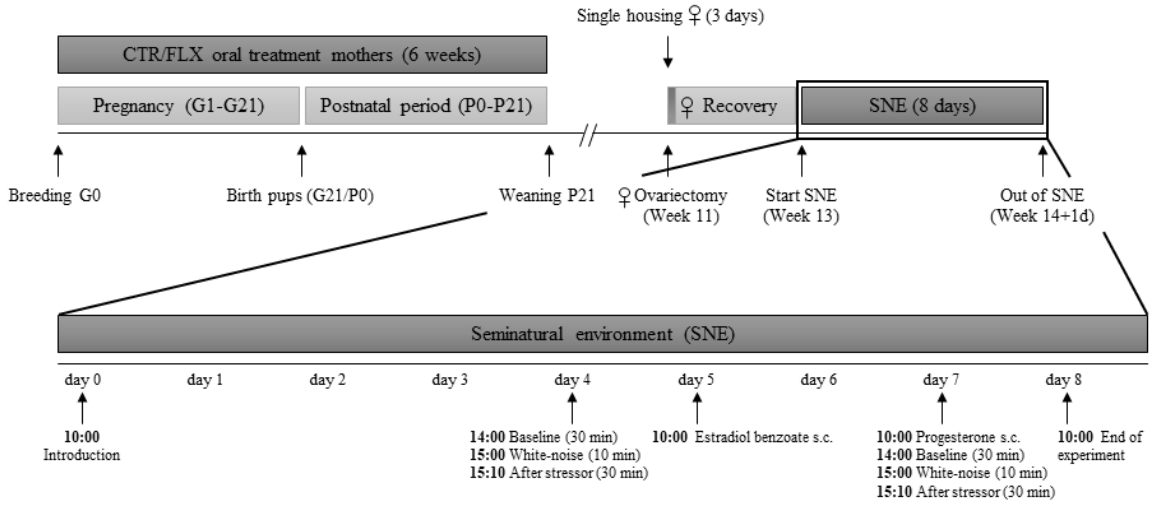


Figure 2: Schematic overview of all experimental procedures. CTR = control, FLX = fluoxetine, G = gestational day, P =postnatal day, SNE = seminatural environment.

2.9 White-noise

To investigate the response of the offspring to a stressful event, they were exposed to loud noise using 90dB white-noise, produced by a white-noise generator (Lafayette instruments, Lafayette, IN) connected to two loudspeakers (Scan-Speak Discovery 10F/8414G10, HiFi Kit Electronic, Stockholm) from which one was placed in the open field and one in the burrow area. Loud noise is often used as stressor in pharmacological and behavioral studies because it produces a strong fear response in rats (Weyers et al., 1994). In addition, white-noise is a similar sound to rattlesnake rattles (Rowe et al., 1986). Since rattlesnakes are predators for rats, this creates immediately a simulation of a natural fear situation. Exposure to white-noise occurred on day 4 (without hormones) and on day 7 (when females were receptive) at 15:00 h and lasted for 10 minutes.

315 **2.10 Behavioral analysis**

316 The frequency and/or duration of a wide variety of behaviors was scored by an observer
317 blind for the treatment of the animals (for the different behaviors, see Table 1). These behaviors
318 were scored on various time points:
319 1). Baseline behavior on day 4 – 30 minutes - the females were without hormones (diestrus)
320 2). Behavior during exposure to white-noise on day 4 – 10 minutes
321 3). Behavior directly after the white-noise on day 4 – 30 minutes
322 4). Baseline behavior on day 7 – 30 minutes - the females were in proestrus and thus sexual
323 receptive
324 5). Behavior during exposure to white-noise on day 7 – 10 minutes
325 6). Behavior directly after the white-noise on day 7 – 30 minutes
326 Baseline behavior was scored on day 4, after which rats had been habituated to their
327 environment (day 0 – day 3) and exploratory behavior was reduced. Baseline observations on day
328 4 and 7 started at 14:00 h and lasted for 30 minutes. This specific time point was chosen because
329 females are most receptive 4 hours after the progesterone injection (on day 7) (Glaser et al.,
330 1983). To keep scoring time points the same, we chose the same time point on day 4 as well.
331 White-noise exposure on day 4 and 7 started at 15:00 h and lasted for 10 minutes. These
332 10 minutes during white-noise, and the following 30 minutes thereafter were scored separately.
333 The frequency and/or duration of a wide variety of behaviors was scored by an observer blind for
334 the treatment of the animals (Table 1). In addition, the location of the animal was scored: in the
335 open field or in the burrow system. During interactions with other animals, the interacting partner
336 was also noted. All behavioral scoring was done using the Observer XT, version 12 (Noldus,
337 Wageningen, the Netherlands). One 30-minute session was scored by 3 independent observers to
338 calculate the interobserver correlation with a Spearman's rho, which turned out to be 0.93.

339 **Table 1. Ethogram of observed behaviors in the seminatural environment**

Behavior	Description
Walking	Walking through the environment
Running	Running with speed through the environment
Walking over/under	Walking over or under another animal
Pursuing	Moving or running forward in the direction of a conspecific
Nonsocial exploration	Exploring the environment by sniffing, usually when slowly walking or sitting still
Resting/immobile alone	Sitting or sleeping with minimal movement of the head without other rats in close vicinity
Resting/immobile socially	Sitting or sleeping with minimal movement of the head with at least 1 other rat on maximum 1 rat body length away
Hiding in shelter alone	Being in the shelter alone
Hiding in shelter socially	Being in the shelter with at least one other rat
Allogrooming	Grooming any part of the partners body, usually on the head or in the neck region
Sniffing anogenitally	Sniffing the anogenital region of the conspecific
Sniffing other rat	Sniffing any part of the conspecifics body, except for the anogenital region
Pouncing	Jumping onto the neck of the partner, usually followed by a nuzzling movement. Usually occurs very short and rapid
Pinning	Usually in response to pouncing, the partner rotates into a supine position, while the other animal is standing over it
Boxing/wrestling	One or both animals are pushing, pawing and grabbing at each other using their forepaws
Nose-off	Facing another rat, usually in a tunnel, resulting in one rat moving forwards and the other backing up
Fighting	Forming a tight ball with another rat, rolling around while biting.
Kicking	Kicking at another rat using the hind paws
Mount	Mounting on the rump of another rat from behind with pelvic thrusting
Intromission	Mounts including penile insertion
Ejaculation	Penile insertion lasts longer than at intromission and is associated with rhythmic abdominal contractions
Paracopulatory behavior	Female approaching a male followed by runaway, often associated with hops, darts, ear wiggling
Lordosis	Receptive behavior with a hollow back and deflect of tail to one side
Carrying nesting material	Playing with or carrying around nesting material
Carrying wood sticks	Playing with or carrying around the wooden stick
Pushing bedding	Moving the bedding material around
Self-grooming	Paw strokes made by the nose and ears, followed by body licking
Postcopulatory self-grooming	Self-grooming immediately after copulation
Eating	Eating, usually while sitting
Drinking	Drinking from one of the bottles in the open field
Freezing	Complete absence of movement in addition to a tense body posture
In opening	Standing in one of the openings connecting the open field and the burrow system and watching to the other side
Rearing	Exploring while raising itself upright on its hind paws
Fleeing	Running away from another rat with high speed
Behavioral clusters of observed behaviors in the seminatural environment	
Activity (non-socially)	Combines walking, walking over/ under, running, pursuing, and nonsocial exploration

Passive behavior	Combines resting alone, resting socially, hiding alone, and hiding
Social context	Combines socially active behavior *and* socially passive behavior
Socially active behavior	Combines pinning, pouncing, sniffing anogenitally, allogrooming, sniffing other rats
Socially passive behavior	Combines hiding socially, and resting socially
Conflict behavior	Combines nose-off, fighting, kicking, and boxing/wrestling

340

341 **2.11 Statistical analysis**

342 As indicated in table 1, behavioral clusters were created beforehand by grouping relevant
343 behaviors. These behavioral clusters and the separate behavioral data from the open field, the
344 burrow system and the total environment were analyzed in different ways. First, the behavior
345 observed on day 4 and 7 were combined and analyzed for the periods “baseline”, “white-noise
346 exposure”, and “after stressor”. Then, the behavior on day 4 and 7 were analyzed separately for
347 the same periods.

348 A Shapiro–Wilk test showed no homogeneity of variance. All behavioral data were
349 therefore analyzed using the nonparametric Mann–Whitney U test to compare FLX-rats with
350 CTR-rats. The Wilcoxon test was used when the different test periods were compared.

351 Since relatively few litters were used, a Kruskal-Wallis test was performed to check for
352 possible litter effects, which were not found.

353

354 **3. Results**

355 Since we explored all the behaviors that the rats performed in the seminatural
356 environment, this experiment generated a lot of data. It is, therefore, impossible to discuss all the
357 behaviors separate in this result section. A complete overview of all the behaviors at the different
358 test moments can be found in Table S2 of the supplementary materials.

359 Most behavioral differences were found in females rats. The difficulty when studying
360 females is that their behavior is largely depending on their estrous cycle phase (hormonal state).

In this experiment, we controlled for the hormonal state and tested them during diestrus (day 4) and during proestrus (day 7), and the data was presented separately. However, analysis of the data in which both days are combined, and thus without taking into account the hormonal state of the female, is maybe most similar to the natural situation in which females can be in different phases of their estrous cycle. Therefore, the description of these results can also be found in the supplemental materials (Results SR1).

3.1 Baseline behavior

First, we were interested in the effects of perinatal FLX exposure on the baseline behaviors in male and female rats compared with CTR-rats. Therefore, we analyzed the behavioral data for 30 minutes on day 4 and day 7 at 14:00 h, during the dark phase.

On day 4, we found that FLX-females were overall less active than CTR-females (Z= -2.495, p= 0.013, d = 1.387, Figure 3A), an effect that was mainly caused by a decrease in nonsocial exploratory behavior in the burrow area (Z= -2.498, p= 0.012, d = 1.403, Figure S3A). In males, on the other hand, no behavioral differences in general activity were found.

In terms of social behavior, we first investigated the effects on all social behaviors together, meaning a combination of social passive (e.g. resting in groups) *and* active social behaviors (e.g. sniffing and grooming behavior towards others) pooled into one parameter called "social context". It was found that both FLX-females (Z= -2.495, p= 0.013, d = 1.292) and FLX-males (Z= -2.344, p= 0.019, d = 1.236) appear to engage in total social behavior more than CTR-rats (Figure 3B). When investigating the type of social behavior (passive versus active) in more detail, it was found that this increase is social behavior was caused by an increase in the amount of time spent on passive social behavior like resting and/or hiding in the vicinity of another rats (females: Z= -2.873, p= 0.004, d = 1.598; males: Z= -2.873, p= 0.023, d = 1.191, Figure 3C). In

385 contrast, FLX-females, but not FLX-males, tended to spend less time on active social interactions
386 (such as sniffing others) in the burrow area than CTR-females (trend: Z= -1.828, p= 0.068, *d* =
387 0.929, Figure 3D).

388 When looking at conflict behavior, even though the total amount of time that was
389 measured in this behavior was limited, FLX-females were for a significantly shorter duration
390 involved in conflicts in the burrow area than CTR-females (Z= -2.097, p= 0.036, *d* = 0.914,
391 Figure S3B). This difference was not found in FLX-males. Another finding that was more
392 pronounced in male rats during baseline measures on day 4, was that FLX-males spent less time
393 grooming themselves compared with CTR-rats (Z= -2.344, p= 0.019, *d* = 0.881, Figure 3E).
394 FLX-females, also groomed themselves slightly less than CTR-females, although this just missed
395 significance (trend: Z=-1.745, p=0.081, *d* = 0.556).

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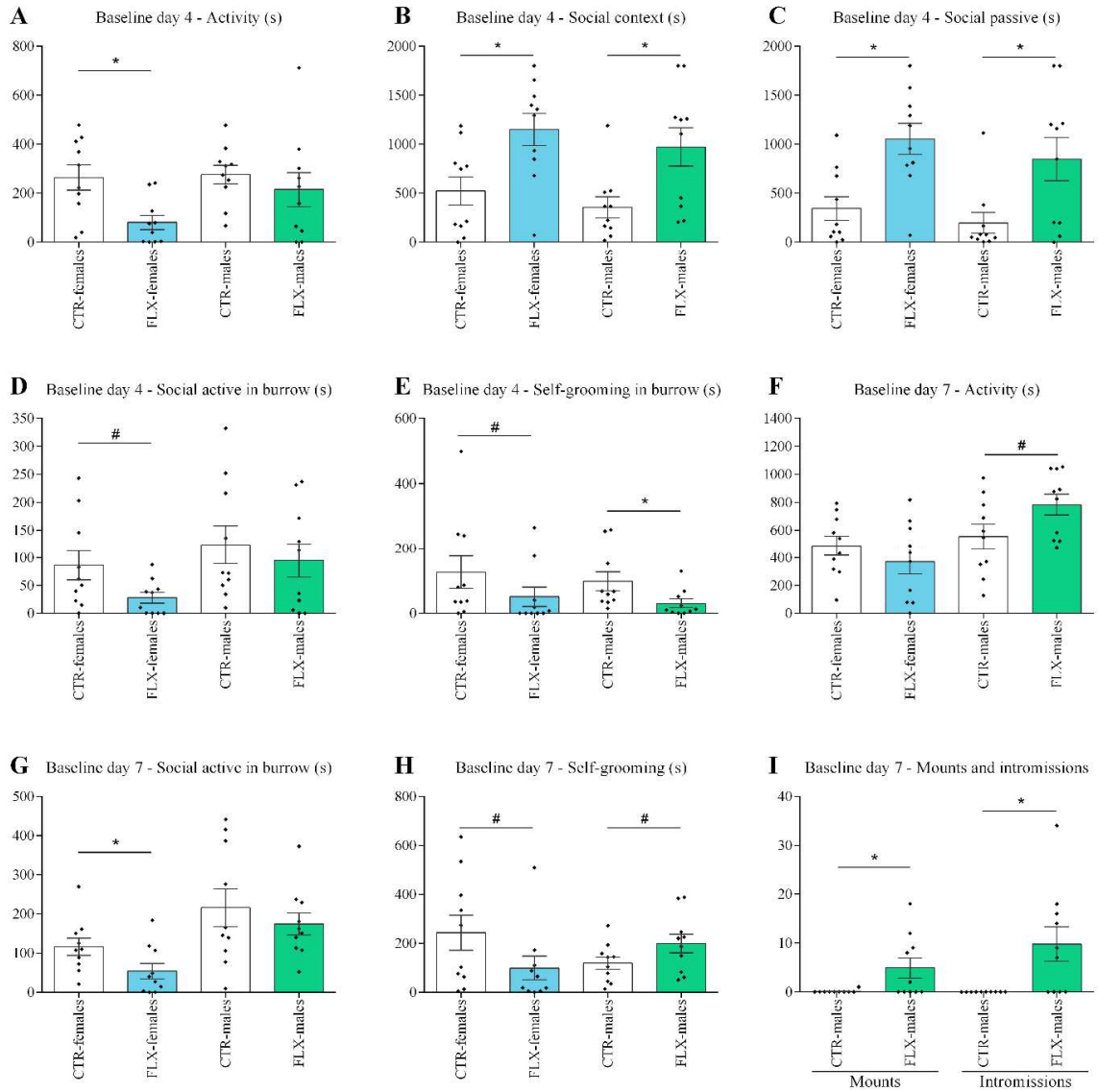
409 **Figure 3. Behavioral baseline effects of perinatal SSRI exposure**



410

411 *Figure 3. The data represents the time spent (s) on each behavior at adulthood in the seminatural*
412 *environment at baseline on day 4 and 7: general activity (day 4) (A), being in a social context*
413 *(day4) (B), being socially passive (day 4) (C), social activity in the burrow area (day 4) (D), self-*
414 *grooming in the burrow area (day 4) (E), general activity (day 7) (F), social activity in the*
415 *burrow area (day 7) (G), self-grooming (day 7) (H), and number of mounts and intromissions*
416 *(day 7) (I). All graphs show the comparison between FLX-females (n=10) and CTR-females*
417 *(n=10), and/or FLX-males (n=10) versus CTR-males (n=10). Data are shown with individual*
418 *data points, with the bars representing the mean±standard error of the mean. * $p<0.05$, # $p<0.1$*
419 *compared with CTR-females or CTR-males.*

On day 7, the females were in menstrual proestrus due to estrogen and progesterone injections on day 5 and 7, respectively. Consequently, they became sexually receptive, which resulted in the display of sexual interactions.Given the background of this intervention, it was found that most behavioral differences present on day 4 baseline were absent on day 7 baseline.

On day 7, we actually found that FLX-females were just as (non-socially) active as CTR-females (Figure 3F). At the same time, FLX-females spent more time being passive than CTR-females (Z=-1.268, p=0.023, d = 1.034, Figure S3C), an effect that was mostly caused by an increase in time spent hiding instead of a difference in socially or solitary resting (as on day 4).

Although FLX-females still spent more time in a social context, this effect was no longer significant on day 7 (Figure S3D). However, a behavior that was still present on day 7 (and comparable/stronger compared to day 4) was the amount of active social behavior: FLX-females had less social interactions than CTR-females in mainly the burrow area (Z= -2.117, p= 0.034, d = 0.996, Figure 3G). In contrast, when we look at the amount of sexual interactions, FLX-females spent less time showing paracopulatory behavior (Z= -2.008, p= 0.045, d = 0.351, Figure S3E), and showed a tendency in receiving less mounts than CTR-females did (trend: Z= -1.819, p= 0.069, d = 0.509; Table S2). Furthermore, FLX-females were pursued by other rats for a shorter duration compared with CTR-females (Z= -2.260, p= 0.024, d = 0.351, Figure S3F). As a consequence, FLX-females also showed fewer lordosis responses than CTR-females (trend: Z= -1.954, p= 0.051, d = 0.610, Figure S3G). In terms of self-grooming, on day 7 during the baseline period, FLX-females also tended to groom themselves less than CTR-females in the burrow area (trend: Z= -1.777, p= 0.076, d = 0.784, Figure 3H).

Now that the females were receptive, FLX-males started to show an interesting pattern of behavior. A trend was found towards an increase in general activity in FLX-males compared to CTR-males (trend: Z= -1.739, p= 0.082, d = 0.810, Figure 3F), mostly seen in the open field (Z=

-1.752, p= 0.08, d = 0.981). This effect was most likely, but not solely, caused by an increase in the amount of time FLX-males spent pursuing other rats in the open field compared with CTR-males (trend: Z= -1.757, p= 0.079, *d* = 0.952, Figure S3H). This pursuing behavior was necessary for the sexual interactions: FLX-males mounted (Z= -2.097, p= 0.036, *d* = 1.048) and intromitted (Z= -2.796, p= 0.005, *d* = 1.253) more often than CTR-males (Figure 3I). At the same time, the sexual behavior induced the display of postcopulatory self-grooming, immediately explaining the higher amount of both the postcopulatory self-grooming (Z= -2.484, p= 0.013, *d* = 1.368, Table S2) and trend in higher amount of self-grooming (trend: Z= -1.663, p= 0.096, *d*= 0.790, Figure 3H) in FLX-males compared with CTR-males. As a logical consequence of the higher activity, there was also a trend that FLX-males were less passive than CTR-males (trend: Z= -1.814, p= 0.07, *d* = 0.930, Figure S3C).

3.2 Behavior during white-noise exposure

Secondly, we were interested in whether perinatal SSRI exposure affects coping with a stressor. Therefore, we exposed the rats to a 10-minute white-noise stressor and measured their behavioral responses during this period. Interestingly, we found that FLX-rats responded in a similar way to the white-noise as CTR-rats on day 4 (Figure 4A-E). The only interesting finding was that FLX-males changed from grooming themselves slightly less than CTR-males in the period before the stressor to grooming themselves now more during the exposure to the white-noise, but they still did not groom themselves more than CTR-males (Z=-1.379, N.S.). FLX-females, on the other hand, groom themselves equally compared with CTR-females (Figure 4E). No significant differences were found between the amounts of time spent freezing upon white-noise exposure (Table S2).

On day 7, we found that again that FLX-rats responded similarly to the white-noise exposure as CTR-rats (Figure 4F/G and Table S2), except for self-grooming behavior. FLX-males groomed themselves extensively more than CTR-males (Z= -2.571, p= 0,01, *d* = 1.351, Figure 4H). No difference in self-grooming was found in the females. In addition, no significant differences were found in freezing behavior (Table S2).

In terms of sexual activity-related behavior, the only relevant different was that FLX-females were still pursued less by other rats during the white-noise episode than CTR-females (Z= -2.097, p= 0.036, *d* = 0.983, Table S2).

Figure 4. Behavioral effects of perinatal SSRI exposure during white-noise exposure

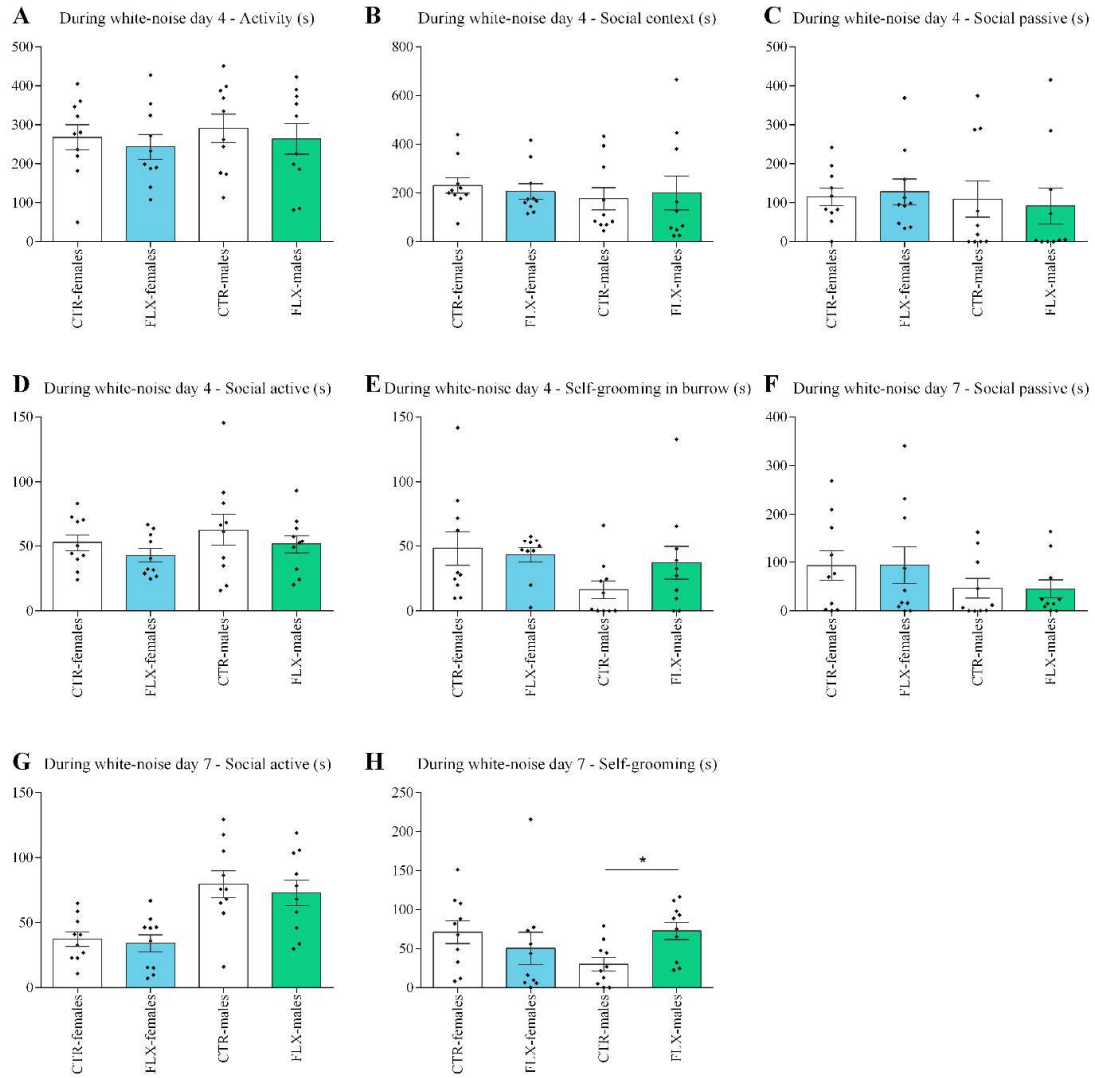


*Figure 4. The data represents the time spent (s) on each behavior at adulthood in the seminatural environment during white-noise exposure on day 4 and 7: general activity (day 4) (A), being in a social context (day 4) (B), being socially passive (day 4) (C), social activity (day 4) (D), self-grooming in the burrow area (day 4) (E), social passive (day 7) (F), social activity (day 7) (G), and self-grooming (day 7) (H). All graphs show the comparison between FLX-females (n=10) and CTR-females (n=10), and/or FLX-males (n=10) versus CTR-males (n=10). Data are shown with individual data points, with the bars representing the mean±standard error of the mean. * $p<0.05$*

3.3 Behavior after the stressor

At last, we were interested in whether FLX-rats are responded different to a stressor compared with CTR-rats and investigated the behavior after the white-noise exposure. We looked at whether behavioral differences from baseline persisted after a stressful event, and/or whether new behavioral variances appeared after the stressor between FLX- and CTR-rats. Therefore, we observed the behavior on day 4 and day 7 at 15:10 h after the stressor, during the dark phase. Our results of day 4 showed that the differences in behavior found at baseline were attenuated after the stressor (Figure 5A-D). We found no differences between FLX-males and females and CTR-males and females in their general activity or their behavior in social context. In terms of active social interaction, FLX-females still seem to spend less time interacting socially compared with CTR-females, but the effect was no longer significant (Z= -1.436, N.S., Figure 5D).

However, when we looked in more detail into the time spent in social context and calculated the percentage of time spent in social passive behavior before and after the stressor, we found that FLX-females actually responded differently to the stressor than CTR-females. As shown in Figure 6, both CTR-females and FLX-females seem to have rats who do not behave differently after a stressor, however, while part of the CTR-females increase the percentage of social resting, a large part of FLX-females clearly decrease their percentage in a social environment (and start resting more solitarily). Although the group was divided, the effect of FLX-females was significantly different (Z=-2.041, p=0.041, *d=0.845*). CTR-males and FLX-males did not show such a different pattern in social passive behavior before and after the stressor.

However, in males, the stressor did seriously affect the self-grooming behavior of FLX-males. We found that the increase in self-grooming behavior that was found during the white-noise period in FLX-males, was strengthened during the period after the stressor. After the

stressor, FLX-males significantly groomed themselves longer than CTR-males (Z= -2.519, p= 0.012, *d* = 1.670, Figure 5E). FLX-males also groomed themselves significantly longer after the stressor compared with baseline (Z=-3,141, p=0.002, *d*=2.156, Figure 7C-D). FLX-females now groomed themselves in a level similar to CTR-females. However, when baseline and after stressor were compared, FLX-females also groomed themselves significantly longer (Z=2.324, p=0.02, *d*=1.122, Figure 7A-B). Lastly, it should be mentioned that FLX-males, compared with CTR-males, were observed freezing for a longer total period of time after white-noise exposure when in the open field (Z= -2.163, p= 0.031, *d* = 0.783, Table S2). Although brief as that was, it is still interesting because four FLX-males show this behavior, whereas none of the CTR-males in the open field were seen freezing.

On day 7, we found again no behavioral differences between FLX-rats and CTR-rats after exposure to the stressor (Figure 5F-H, TableS1). The only difference we found was the increased levels of self-grooming in FLX-males on day 7 after the stressor, although it did not reach significance in the amount of time spent on it, but only in the number of self-groom episodes (Z=-2.091, p= 0.037, *d* = 0.987, Table S2). In addition, FLX-males continued copulating: FLX-males had more intromissions than CTR-males (Z= -2.163, p= 0.031, *d* = 0.799, Figure 5I), although this effect was caused by only 3 copulating males. However, interestingly, the copulatory behavior were now mostly performed in the burrow area instead of in the open field. Indicating that the stressor affected the location in which copulation takes place. FLX-females, on the other hand, now spent an equal amount of time on sexual activity as CTR-females (Table S2).

Figure 5. Behavioral effects of perinatal SSRI exposure after a stressor

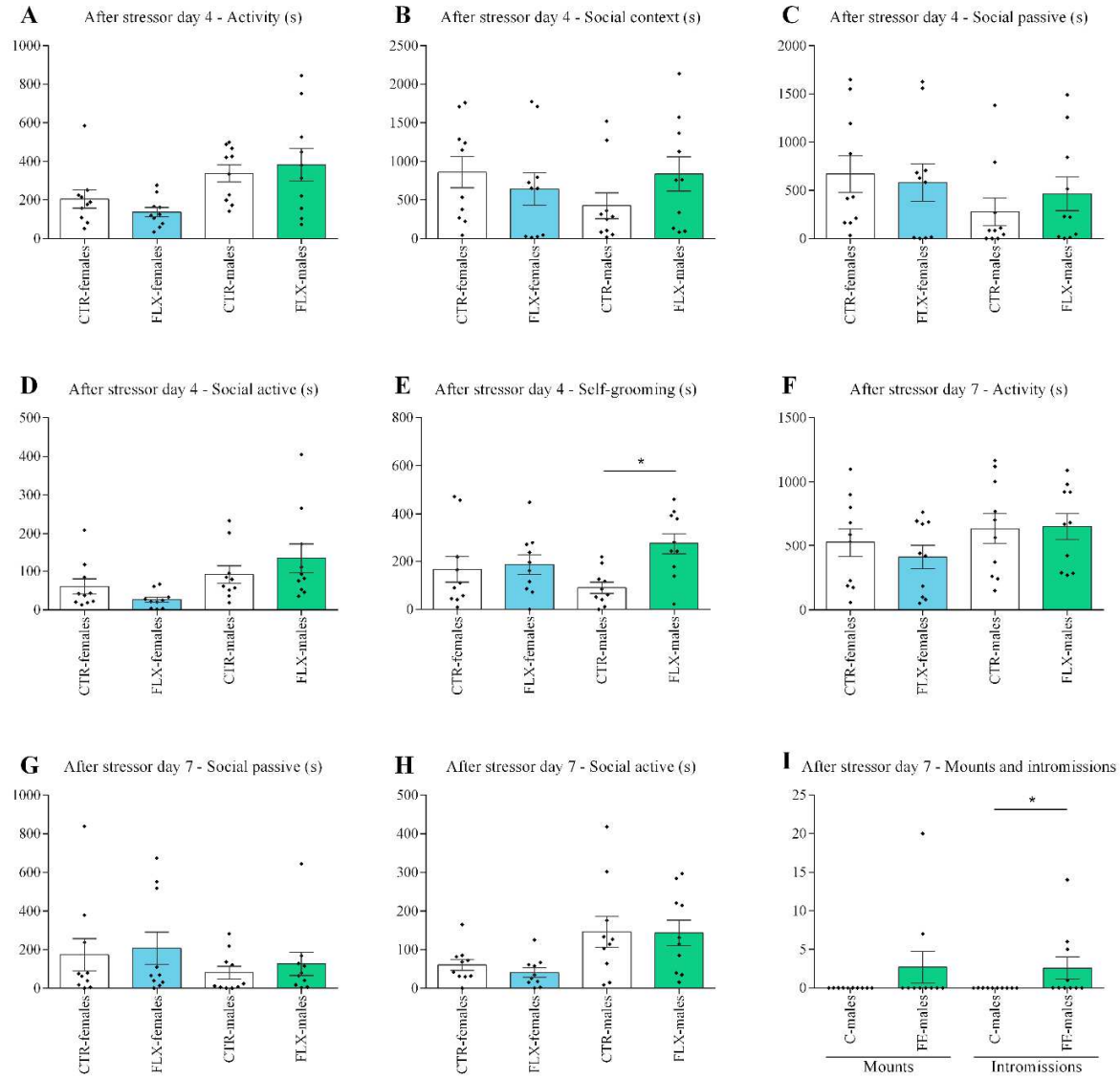
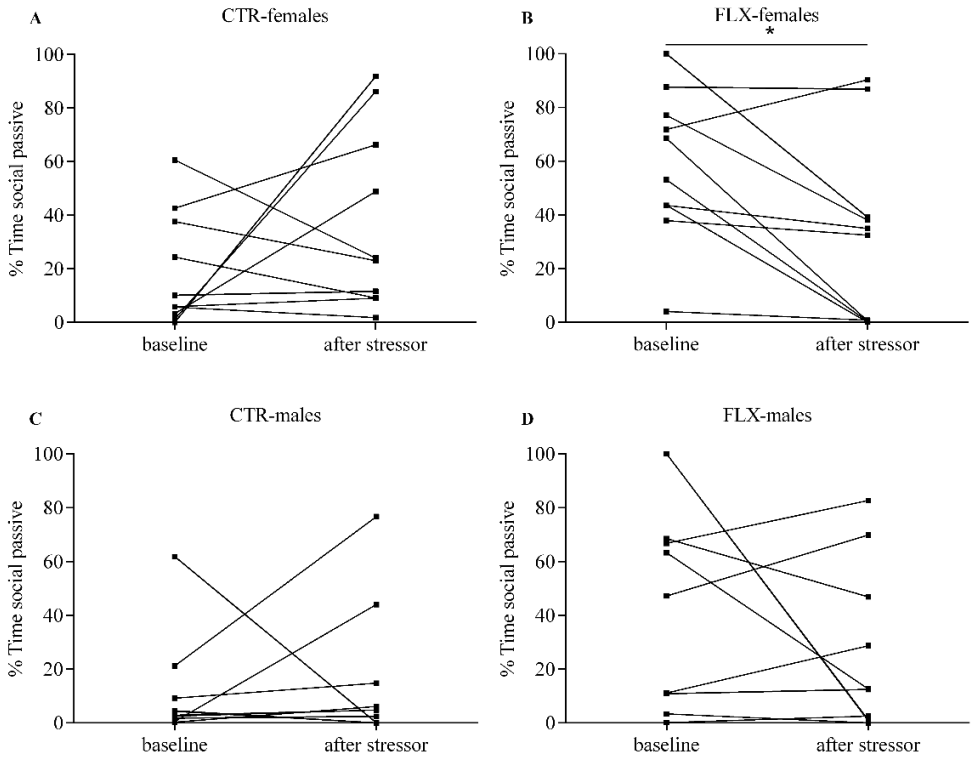


*Figure 5. The data represents the time spent (s) on each behavior at adulthood in the seminatural environment after a white-noise exposure on day 4 and 7: general activity (day 4) (A), being in a social context (day 4) (B), being socially passive (day 4) (C), social activity (day 4) (D), self-grooming in the burrow area (day 4) (E), general activity (day 7) (F), social passive (day 7) (G), social active (day 7) (H), and number of mounts and intromissions (day 7) (I). All graphs show the comparison between FLX-females (n=10) and CTR-females (n=10), and/or FLX-males (n=10) versus CTR-males (n=10). Data are shown with individual data points, with the bars representing the mean±standard error of the mean. * $p<0.05$*

562 **Figure 6. Difference in percentage of time spent on being social passive**



563

564 *Figure 6. The data represents the percentage of time rats spent on being socially passive. All*
565 *graphs show the comparison between baseline and after stressor of CTR-females (n=10, A),*
566 *FLX-females (n=10, B), CTR-males (n=10, C), and FLX-males (n=10, D). Data are shown in*
567 *individual data points, with the lines connecting the same individuals at baseline and after*
568 *stressor. * $p<0.05$*
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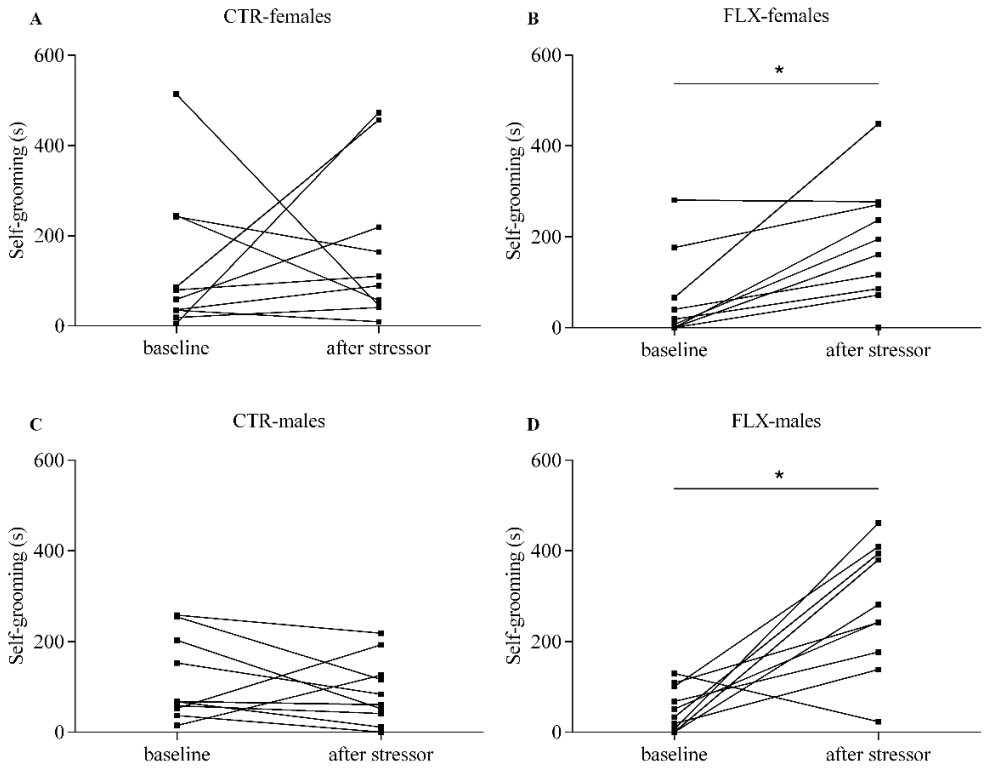
577 **Figure 7. Difference in time spent grooming on baseline and after stressor**



578

579 *Figure 7. The data represents the time spent (s) on grooming themselves at adulthood in the*
580 *seminatural environment on day 4: CTR-females (A), FLX-females (B), CTR-males (C), and FLX-*
581 *males (D) Data are shown in individual data points, with the lines connecting the same*
582 *individuals at baseline and after stressor. * p<0.05*
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4. Discussion

In the present study we sought to study the effects of perinatal SSRI-exposure on neurobehavioral outcomes in adult offspring using a seminatural environment, allowing us to control environmental factors and observe the full behavioral repertoire of the animals in a more naturalistic setting.

Our findings indicate that perinatal SSRI exposure can induce behavioral adaptations. Rats that were exposed to SSRIs during early development show at baseline a lower general activity at adulthood than control rats, which was mostly explained by a decrease in nonsocial exploration. In terms of social behavior, our data surprisingly showed that fluoxetine-exposed rats seek more social contact than control rats. This increased sociability, however, is a result of more passive behavior performed in a social context in both males and females. In contrast, female rats that were perinatally exposed to SSRIs tended to show less active social behaviors than control females.

When exposed to a stressful event, presented as white-noise, all rats responded similarly, resulting in an attenuation of the pre-stressed found alterations. However, when the behavior following the stressor was investigated in more detail, it was found that FLX-rats changed preference from resting in groups to more solitary resting, whereas control rats actually started to seek a more social (passive) environment. In addition, FLX-males started to self-groom themselves extensively more than before presentation of the stressor, even more than control rats. The FLX-males also showed increased freezing behavior in the open area compared to control rats.

It should be mentioned that most of the behavioral differences were found on day 4 when the females had not received any hormonal treatment and were sexually non-receptive, while no differences were found on day 7 when the females were hormonally primed and sexually

receptive. At first sight, this could indicate that the hormonal state of the females plays an important role in the expression of behavioral effects of perinatal SSRI exposure. However, an alternative explanation could be found in the fact that the females are now in their behavioral estrus and receptive for sexual interactions. Both males and females could thus be occupied by the opportunity to copulate and thereby show normal behavioral outcomes. If so, the lack of effects due to perinatal SSRI exposure during proestrus in females does not necessarily have to be a result from the hormones themselves.

4.1 Social behavior

4.1.1 Social behavior at baseline

We found that both FLX-males and FLX-females spent passive moments more often in the company of another rat (social resting) compared with CTR-rats. When both the passive and active behavior performed in a social context are analyzed as total social behavior, we found (on day 4) that fluoxetine exposure during development induced a phenotype in adulthood in which the rats are more social than control rats. This finding is in line with a recent study by Gemmel et al. that similarly treated dams with 10 mg/kg fluoxetine throughout most part of pregnancy and until weaning of the pups. Adult females from fluoxetine treated dams increased their social investigation time with another female, while adult males increased their play behavior (Gemmel et al., 2019). Furthermore, Ko et al. found that injecting male rat offspring directly with fluoxetine from PND0-4 increased their sniffing, contact and total interaction behavior with a conspecific when adult (Ko et al., 2014). In contrast, a study by Olivier et al. showed that prenatal SSRI exposure in male rats decreased the amount of time spent on social exploration behavior measured by sniffing and grooming others (Olivier et al., 2011b). Furthermore, we recently showed that fluoxetine treatment from G0 till PND21 resulted in decreased social interaction in

male but not female rats (Houwing et al.,2019b). Likewise, postnatal SSRI exposure affected social exploration time in social preference tests in which the amount of exploration time to a conspecific was compared with the time spent sniffing a novel object. Male and female offspring (postnatally treated with SSRIs) show decreased conspecific exploration compared with novel object exploration at both juvenile and adult age (Khatri et al., 2014; Rodriguez-Porcel et al., 2011; Simpson et al., 2011; Zimmerberg and Germeyan, 2015). Similarly, the majority of studies studying specifically social play behavior in rats found a decrease in social play as a result of early SSRI exposure (Khatri et al., 2014; Olivier et al., 2011b; Rodriguez-Porcel et al., 2011; Simpson et al., 2011). However, we did not study the highly playful juvenile rat but social interaction at adulthood and we barely observed play behavior at our chosen time points. In the present study, we found a tendency towards decreased active social behavior in FLX-females, but not in males. Since male rats are consistently less socially active in all the other studies, the fact that we were unable to replicate this finding, can most likely be ascribed to the test setting. Our study used a seminatural environment, which allowed us to study all behaviors expressed by the rats at the same time, meaning that the rats have the freedom to perform any behavior at any time point they want. One should also note that the basal behavior was observed at day 4, when exploration behavior was reduced (compared with day 0), and rats were no longer unfamiliar to each other. This might have influenced the findings in the present study as well. Differences of acute novel social interactions may still exist and this remains to be investigated. In a study of Gemmel et al 2017, it was shown that social play behavior in juvenile rats exposed to fluoxetine during development was increased when paired with an unfamiliar partner, while they found no differences in social play when interacting with their siblings (Gemmel et al., 2017). These data may confirm the theory that novel acute social interactions may have a different outcome when

comparing the already established social interactions such as the observations in the seminatural environment on day 4 with social interactions with siblings in their home cage.

4.1.2 Social behavior after stressor

With the additional exposure to a 10-minute white-noise stressor within this environment, we were able to investigate the acute and long-term behavioral responses to this novel and stressful stimulus, and the behavioral consequences afterwards. At first it seemed that the alterations in social behavior disappeared, but after a more detailed analysis, we found that FLX-rats actually respond differently to the stressor than CTR-rats. While FLX-rats were significantly longer passive in a social context at baseline levels compared with CTR-rats, the FLX-females started to rest less in groups (Figure 6) and more solitarily after the stressor. CTR-females, on the other hand, started to rest more in a social context. This suggests that FLX-females have the opposite response in a stressful situation than CTR-females. More research is needed to find out whether this effect is only temporary, will sustain or exacerbates over time, and whether this is alteration is advantageous or disadvantageous before serious conclusions can be drawn.

4.2 Other responses to stressor

During the actual period of white-noise exposure, both CTR- and FLX- rats were more generally active and showed more freezing behavior than baseline. However, overall our data showed that FLX-rats did not differ in their behavior from CTR-rats during the white-noise exposure. Despite the slight increase in the occurrence of freezing behavior, all rats spent the same amount of time freezing. Other studies, on the other hand, have shown that rats exposed to SSRIs during early development responded with exaggerated freezing (or sometimes measured as immobility time) to a novel tone compared with control rats (Khatri et al., 2014; Rodriguez-

Porcel et al., 2011; Simpson et al., 2011). In addition, this increase in immobility lasts longer in FLX-rats than in CTR-rats (Rodriguez-Porcel et al., 2011), suggesting that early developmental SSRI exposure induces hyperreactivity towards a novel auditory stimulus. Our data, however, does not confirm these findings. In addition, it showed that the rats in the seminatural environment, instead of showing a freezing response, started to explore and run through the burrow area more. During the 10 minute of white-noise exposure, the rats actually spent the relative same amount of time running and exploring the burrow as during the 30 minute baseline period (about a 3-fold increase). This increase in general activity is most likely a stressful response to the white-noise. The lack of effect on freezing behavior in our paradigm, on the other hand, suggests that rats respond differently to novel auditory stimuli in a seminatural environment than in a small test setting. One explanation could be that the social environment creates a kind of social buffering: the presence of familiar conspecifics have positive comforting effects in stressful situations (Kiyokawa et al., 2014; Terranova et al., 1999). Another or additional explanation, however, could again be found in the fact that rats can express all kind of behaviors in a seminatural environment, which is at the same time their home cage and test environment. While freezing is the most logical behavior in a small test set-up in response to a stressor, this behavior is not needed in large and familiar living spaces where one could just as well escape from the stressor or danger by walking away. Whatever the reasons are behind the lack of freezing behavior, our data clearly showed that FLX-rats do not respond differently to a stressor, in terms of freezing or exploratory behavior, compared with CTR-rats.

4.3 Stress-coping behavior

Simultaneously, another important change in response to the stressor was observed: a difference in stress-coping behavior in FLX-males when compared with CTR-males. While FLX-

704 males on day 4 groomed themselves significantly less than CTR-males at baseline, they started to
705 self-groom more during, but especially after, the white-noise exposure (Figure 7). FLX-females
706 also groomed themselves relatively more after the stressor, but the time spent on this behavior
707 was not different from CTR-females. As discussed in (Smolinsky et al., 2009), grooming is an
708 important behavior observed in many species serving several functions. Beyond the most obvious
709 purpose of hygiene, grooming is also performed for stimulation of the skin, thermoregulation,
710 chemo-communication, social interaction, de-arousal, and stress reduction (Sachs, 1988; Spruijt
711 et al., 1992; Terry, 1970). In rodents, this grooming behavior is rather patterned and starts with
712 licking of the paws, followed by washing the nose and face, the head, the body, the legs, and
713 finally licking the tail and genitals (Fentress, 1988; Smolinsky et al., 2009). In addition, grooming
714 is highly sensitive to various stressors, psychotropic drugs and genetic manipulations, making it
715 an important player in behavioral adaptation to stress, including stress-coping and de-arousal
716 (Choleris et al., 2001; Dunn et al., 1987; Spruijt et al., 1992). In fact, grooming can be interpreted
717 as a typical displacement behavior in which an animal is in conflict to perform two or more
718 different behaviors and where the response is a displacement activity that is usually unrelated to
719 the competing behaviors. This stress-induced displacement grooming, however, is ethologically
720 different from low-stress comfort grooming, indicating that the amount of grooming behavior by
721 itself is insufficient as a measure for stress. Interestingly, differences in grooming patterns in low
722 and high stress situations have been studied. Whereas low-stress comfort grooming occurs
723 spontaneously as a transition between rest and activity, and usually follows an uninterrupted
724 pattern of the order described above, high stress levels induce more frequent and rapid short
725 bouts of interrupted less patterned activity of self-grooming (Kalueff and Tuohimaa, 2004, 2005).
726 These differences in grooming pattern, or microstructures, could be used as indicators for
727 different neuropsychiatric disorders (Kalueff et al., 2016): e.g. obsessive compulsive behavior

and autistic phenotypes could be related with high locomotor, but rigid patterned grooming, while anxiety would be represented by high locomotor, but more flexible patterning. Depression, on the other hand, could result in a self-groom microstructure of low locomotor activity with a slight patterned grooming (Kalueff et al., 2016).

Unfortunately, due to the fact that our seminatural environment is rather large, our video images did not have the right resolution to study the self-groom patterns in more detail. Still, the difference in self-groom behavior before and after the stressor makes it plausible to believe that the rats performed different patterns of self-grooming reflecting less comfort/hygiene grooming at baseline, compared with higher levels of stress-coping grooming after the stressor. Our baseline data is in line with a previous finding in which perinatal SSRI exposure reduced the time in which males groomed themselves during a social behavior test (Olivier et al., 2011b). At the same time, the increased levels of self-grooming coincide with the finding that FLX-rats show increased burying behavior in a marble burying test which is used to study repetitive and perseverative behavior (Sprowles et al., 2017). As a result, we hypothesize that perinatal SSRI exposure changes the stress-coping mechanisms in male rats at adulthood after the exposure to stressors. Future research should clarify whether the higher activity of grooming behavior reflects in the direction of anxiety-related versus repetitive compulsive self-grooming.

4.4 Aggressive behavior

In the seminatural environment, many more behaviors can be explored such as aggressive and sexual behaviors. Previous studies have shown that perinatal SSRI exposure increases aggressive behavior in adult male mice (Kiryanova and Dyck, 2014; Svirsky et al., 2016). However, we recently showed that fluoxetine treatment during gestation and the postnatal period reduced the offensive behavior of male rats in a resident-intruder test set-up (Houwing et al., in

752 preparation). In the present study, however, FLX-males spent the same amount of time in conflict
753 situations as CTR-males, while FLX-females seem to show less conflict behaviors on day 4. We
754 would, however, not dare to draw any serious conclusions based on this observation, because on
755 average the rats do not spend more than 40 seconds on this agonistic behavior. Wistar rats are
756 known to show low levels of aggressive behavior in general (Koolhaas et al., 2013), and in our
757 seminatural environment set-up the rats do not really have to compete for resources. Drinking
758 water and food pellets were available ad libitum, and even during the period of behavioral estrus
759 there were enough receptive females available for mating. It is, therefore, fair to say that our
760 experimental design was not sufficient for the exploration of aggressive encounters.
761
762 *4.5 Sexual behavior*
763 Also in terms of sexual behavior, our set-up had its limitations. Although we found an
764 increase in copulatory behaviors in FLX-males, previous studies have shown conflicting results
765 of early life SSRI exposure on sexual behavior. Postnatal fluoxetine exposure has been shown to
766 decrease the amount of mounts, intromissions and ejaculations, just as reducing the level of
767 sexual motivation in male rodents (Gouvea et al., 2008; Harris et al., 2012; Rayen et al., 2013;
768 Rodriguez-Porcel et al., 2011). Prenatal SSRI exposure, on the other hand, did not affect male
769 copulatory behavior (Cagiano et al., 2008; Olivier et al., 2011b). In a study we performed before,
770 male rats exposed to fluoxetine during the whole gestational and postnatal period (until weaning)
771 displayed a reduction in the number of mounts compared with control males, but only when the
772 males were sexually experienced (Houwing et al. in preparation). In the FLX-females of the
773 present study, we found a slight decrease in sexual behavior compared with CTR-females. Other
774 studies, however, found a stimulatory effect on paracopulatory and receptive behaviors of
775 postnatal fluoxetine exposure (Rayen et al., 2014). One could argue that the timing of the SSRI

exposure could explain our differences in results, but a better explanation could be found in the fact that we only observed 30 minutes twice. A study by Chu and Ågmo performed in the seminatural environment taught us that the behavioral estrus of female rats can last up to eleven hours, with an average of 7 hours (Chu and Agmo, 2014). During this whole period, male and female rats continue to participate in copulatory behavior until the estrus period ends (Chu and Agmo, 2014, 2015). Male rats seem to copulate in copulatory bouts, defined as the time between the initial mount or intromission and the beginning of a period of sexual inactivity lasting for more than 60 min. When males copulate with naturally cycling females, they have on average about 4±1 bouts during the time they are in the seminatural environment. No such detailed studies have been performed in the seminatural environment with ovariectomized and hormonally primed females, but we can assume that males will in this case copulate in bouts as well. This indicates that we might have observed a time slot in our experiment in which most of the CTR-males might coincidently have been in a break between the copulatory bouts, whereas six out of ten FLX-males were observed within their copulatory bout. As a consequence, it would be very interesting to investigate the differences in behavioral patterns between FLX-rats and CTR-rats during the behavioral estrus period in more detail. This interesting data, however, would be quite substantial, and therefore better suitable for a separate manuscript.

4.6 Affective behavior

In our seminatural environmental approach, we cannot directly relate certain behaviors to the traditional tests, but an indication of anxiety in the seminatural environment might be reflected by visiting the open area less and by more freezing in response to a white-noise stressor. Our results indicated that FLX-rats were present in the open area just as long as CTR-rats, also after the stressful white-noise exposure. In addition, we only observed a slight increase in

freezing after the stressor when FLX-males visited the open area, caused by 4 out of 10 FLX-males. If these parameters would be a measure of anxiety-related behavior, it suggests that perinatal SSRI exposure does not or slightly increases the risk for anxiety-like behavior in adulthood. As mentioned before, the lack of clear anxiety-related behavior could also be explained by the social environment in which our rats were housed. The anxiety traits could be possibly suppressed in a more natural situation in which more behavioral escapes are an option, but come to the surface when exposed to an unnatural unfamiliar situation, or when assessed in acute stressful situations.

4.7 The translational value of the seminatural environment

Altogether, we believe that the seminatural environment is a good approach to study the effects of perinatal SSRI exposure (and other interventions) on naturally expressing behaviors. As mentioned before, the advantage of the seminatural environmental approach is that one can study a wide variety of behaviors at the same time, but in addition one can relate this behavior with other behaviors (e.g. sexual, aggressive, locomotor, and freezing) that are performed within the same setting/experiment. This is on one hand beneficial to the interpretation of the behavioral changes, because it provides additional information about the context of the behaviors, and on the other hand it permits to study several traits of psychiatric disorders at once in a natural situation. To give an example, the seminatural environmental approach allows for exploring several phenotypes often experienced by depressive persons, like reduced general activity, lack of interest in the environment, and limited social contact (social withdrawal). Before someone can be diagnosed with depression, the patient should have first of all characteristics of several traits corresponding to the disorder. But at the same time, these symptoms should cause significant distress or impairment in social, occupational, or other important areas of functioning, meaning in

824 daily life. The seminatural environmental approach allows us to evaluate this aspect as well.
825 Therefore, we believe that the seminatural environment is a valuable test set-up, and has
826 additional advantages compared with the traditional test methods and perfectly suits to study the
827 behavioral outcomes due to SSRI treatment during development.
828
829 4.8 Limitation of this study
830 In the present study, we investigated the effects of fluoxetine exposure in offspring from
831 healthy dams. However, in humans SSRI treatment during pregnancy and lactation is only used
832 in mothers with psychopathologies. Even though exposing offspring from healthy dams to
833 fluoxetine is of use to dissociate the effects of the SSRI from the maternal depression, looking at
834 SSRI exposure in offspring from stressed dams would be more clinically relevant. We recently
835 showed that fluoxetine treatment in healthy dams resulted in reduced social play behavior in male
836 and female offspring (Houwing et al., 2019b), while male, but not female, offspring from an
837 animal model of maternal vulnerability (Houwing et al., 2019a) showed reduced juvenile play
838 behavior similar to offspring from fluoxetine treated healthy dams. Other studies showed that
839 perinatal fluoxetine treatment can prevent reductions in rat juvenile social play behavior caused
840 by pre-gestational maternal stress (Gemmel et al., 2017). Also, perinatal SSRI exposure in
841 healthy dams resulted in reduced copulatory behaviors in male offspring, while male offspring
842 from stressed dams were unaffected (Rayen et al., 2013). Interestingly, SSRI exposure in female
843 offspring facilitated copulatory behaviors, regardless of maternal stress (Rayen et al., 2014).
844 Thus, using an animal model of maternal depression and/or stress has an added value for future
845 studies investigating effects of perinatal fluoxetine exposure in the seminatural environment.
846
847

5. Conclusion

Overall, we conclude that perinatal SSRI exposure causes adaptations in social and stress-coping behaviors at adulthood. FLX-females are mostly affected by reduced general activity and both males and females show altered social behavior. Exposing the animals to a stressor resulted in a different social strategy in FLX-females, and an altered stress-coping behavior in mainly FLX–males. This indicates the existence of sex differences in the responses to SSRI exposure during early development. Whether the adaptations found due to perinatal SSRI exposure are beneficial or disadvantageous remains to be investigated. We show that SSRI exposure during development can have long-lasting effects. However, the SSRIs in our study were administered to healthy dams. Using an animal model of depression instead would improve the clinical relevance. This would make the research more translational to the human situation in which only depressed mothers use antidepressants. In this study we used the seminatural environment and showed it is an excellent tool to study the behavioral adaptations caused by perinatal SSRI exposure (or other interventions) in order to provide better information of the relevance of these changes for the risk for psychiatric disorders.

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872

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1052

Highlights

- Perinatal FLX exposure increased social behavior in both males and females.
- FLX-females show changed social strategy after stressor
- FLX-males show changed stress-coping behavior after stressor
- The seminatural environment is an excellent tool to study behavioral adaptations